Lipolysis Assay Kit for 3T3-L1 Cells Detection of Both Free Glycerol and Non-Esterified Fatty Acids

CAT# LIP-3-L1; LIP-3-NC-L1

INSTRUCTION MANUAL ZBM0042.03

STORAGE CONDITIONS

- **96-well plate cultured 3T3-L1 preadipocytes (LIP-3-L1)** 37°C incubator
- **Reagents & Buffers:** 4°C **Use reconstituted Glycerol Reagent A within 7 days.**
- **Vehicle & Controls:** -20°C

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ORDERING INFORMATION AND TECHNICAL SERVICES

- **Zen-Bio, Inc.**
- **3200 Chapel Hill-Nelson Blvd., Suite 104**
- **PO Box 13888**
- **Research Triangle Park, NC 27709**
- **Telephone** (919) 547-0692
- **Facsimile (FAX)** (919) 547-0693
- **Toll Free** 1-866-ADIPOSE (866)-234-7673
- **Electronic mail (e-mail)** information@zen-bio.com
- **World Wide Web** http://www.zenbio.com

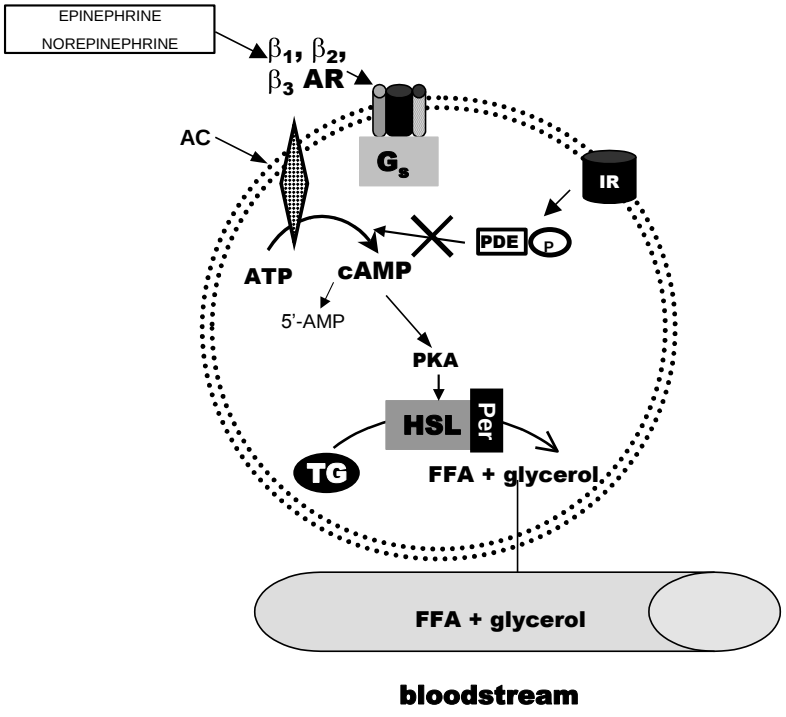
INTRODUCTION

Lipolysis plays a central role in the regulation of energy balance. Lipolysis is the process in which triglycerides (TG) are hydrolyzed into glycerol and free fatty acids. This process releases free fatty acids (FFA) into the bloodstream where they may be either re-esterified by the adipocyte or travel to other tissues and exert other effects throughout the body. Elevated adipocyte lipolysis has been observed in obese and diabetic individuals (Arner 1996). Excessive free fatty acid production is believed to contribute to insulin resistance in skeletal muscle that is observed in obesity. Hormone sensitive lipase is the rate-limiting enzyme catalyzing triglyceride breakdown. Perilipins, one of the PAT (perilipins, adipophilin, TIP47 proteins) family of lipid-associated proteins, are implicated in adipocyte lipolysis by mediating the interaction of HSL with the triacylglycerol molecule (Brasaemle *et al.* 2004; reviewed in, Tansey *et al. 2004.)* The presence of these proteins corresponds to lipolytic stimulation in cultured adipocytes (Braemle *et al.* 2004).

The sympathetic nervous system also plays a key role in the regulation of lipid mobilization. The main lipolytic pathway involves beta-agonists (β-agonists), which activate β-adrenergic receptors via the intracellular G_s proteins in adipocytes. This leads to the activation of adenylate cyclase (AC), which then increases cyclic AMP (cAMP) levels. Elevated cAMP acts as a second messenger to activate hormone sensitive lipase (HSL). HSL, the rate-limiting enzyme regulating adipocyte lipolysis, then catalyzes the hydrolysis of triglycerides and results in the release of glycerol and FFA (increased lipolysis). Phosphodiesterases (PDE) are enzymes that hydrolyze cAMP to 5'-AMP (5 prime adenosine monophosphate). This action results in a decrease in lipolysis. PDE inhibitors increase intracellular cAMP levels. 3-isobutyl-1-methylxanthine (IBMX), a non-specific inhibitor of cAMP phosphodiesterases (PDE), is used as the positive control if your test compounds are suspected PDE inhibitors. Isoproterenol, a non-specific β-adrenergic agonist is used as the positive control if your test compounds affect lipolysis via β-adrenergic receptors (Robidoux *et al*. 2004).

This lipolysis assay kit provides the tool to study chemical compounds that may influence lipolysis in cultured human adipocytes.

Figure 1. Overview of adipocyte lipolysis



ABBREVIATIONS:

AC	adenylate cyclase
AR	adrenergic receptors
G_s	G protein coupled receptor
FFA	free fatty acids
PKA	protein kinase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
IR	insulin receptor
PDE	phosphodiesterase
TG	triglyceride

PRINCIPLES OF THE ASSAYS

Detection of Free Glycerol

Assessing lipolytic activity by the measurement of glycerol released into the medium. Glycerol released to the medium is phosphorylated by adenosine triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5'-diphosphate (ADP) in the reaction catalyzed by glycerol kinase. G-1-P is then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). A quinoneimine dye is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine (4-AAP) and sodium N-ethytl-N-(3-sulfopropyl)m-anisidine (ESPA) with H_2O_2, which shows an absorbance maximum at 540nm. The increase in absorbance at 540nm is directly proportional to glycerol concentration of the sample.

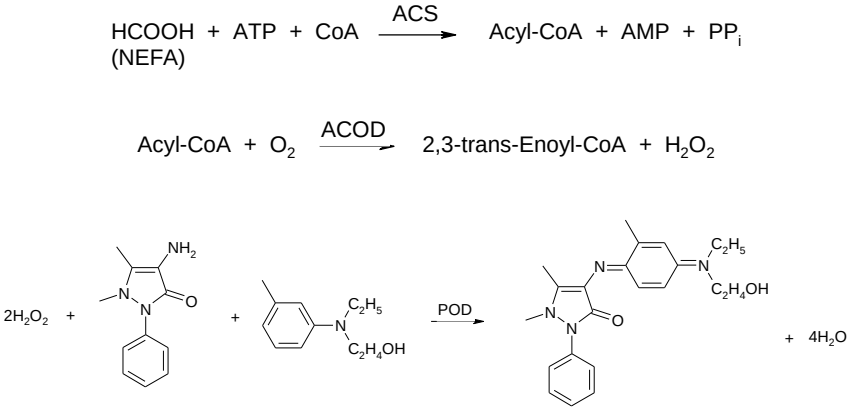
GLYCEROL + ATP ⟶ G-1-P + ADP

G-1-P + O_2 ⟶ DAP + H_2O_2

H_2O_2 +4-AAP + ESPA ⟶ Quinoneimine dye + H_2O

Detection of Non-Esterified Fatty Acids (Free Fatty Acids; FFA)

Assessment of lipolytic activity can also be detected through a coupled reaction to measure non-Esterified fatty acids (NEFA) released by adipocytes. The initial step, carried out by acyl-CoA synthetase (ACS), produces fatty acyl-CoA thiol esters from the NEFA, ATP, Mg, and CoA in the reaction. The acyl-CoA derivatives react with oxygen in the presence of acyl-CoA oxidase (ACOD) to produce hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine which forms a purple product that absorbs light at 550nm. This allows the concentration of NEFA to be determined from the optical density measured at 540 - 550nm.



NOTE:

3 fatty acid molecules are released per triglyceride molecule resulting in a 3:1 fatty acid to glycerol concentration.

ITEMS INCLUDED IN THE KIT _____

ITEM	DESCRIPTION	Cap Color	UNIT	QTY	STORAGE
Plate A	96 well plate 3T3-L1 preadipocytes (LIP-1-L1 ONLY)	---	PLATE	1	37°C
Assay Plates	96-well assay plate, blank	---	PLATE	3	-----
Preadipocyte Medium	3T3-L1 Preadipocyte Medium (cat# PM-1-L1); 50ml (LIP-3-L1 ONLY)		BOTTLE	1	4°C
Differentiation Medium	3T3-L1 Adipocyte Differentiation Medium (cat# DM-2-L1); 15ml (LIP-3-L1 ONLY)		BOTTLE	1	4°C
Adipocyte Medium	3T3-L1 Adipocyte Maintenance Medium (cat# AM-1-L1); 100ml (LIP-3-L1 ONLY)		BOTTLE	1	4°C
Wash Buffer		---	50ML	1	4°C
Vehicle	0.1% DMSO in LIP-2/3 Assay Buffer	PURPLE	1 ml / VIAL	1	-20°C
Positive control	Isoproterenol, 10 mM in DMSO. Dilute to 1 μM in Assay Buffer before use! (i.e.1 μl in 10 ml Assay Buffer)	BLUE	10 μl / VIAL	1	-20°C
Glycerol Reagent A (cat# RGTA-10)	Reconstitute with 11.0 ml deionized water prior to use. Use reconstituted Reagent A within 7 days.	---	BOTTLE	1	4°C
Tray	For multi-channel pipetters, clear polyvinyl		EACH	4	-----
Glycerol standard	Glycerol @ 1mM [Dilute with 400 μl Wash Buffer to make the 200 μM glycerol standard; see page 8 for recommended dilution scheme]	ORANGE	100 μl / VIAL	1	-20°C
LIP2/3 Assay Buffer	100ml	---	100ML	1	4°C
FFA Standard (cat# FFA-STAN)	1mM Stock. See page 7 for standard curve preparation	AMBER	100 μl / VIAL	1	4°C
FFA Diluent A		YELLOW	10.5ML	1	4°C
FFA Diluent B		PINK	5.5ML	1	4°C
FFA Reagent A	Reconstitute using 10.5 ml FFA Diluent A. Discard remainder after 10 days	YELLOW	BOTTLE	1	4°C
FFA Reagent B	Reconstitute using 5.5 ml FFA Diluent B. Discard remainder after 10 days	PINK	BOTTLE	1	4°C

Other equipment/reagents required but not provided with the kit:

- Multi-channel Pipet , single channel pipet and pipet tips
- Sterile trays for multi-channel pipetters during differentiation of cells
- Plate reader with a filter of 540 nm
- Incubator at 37^{o}C
- Large gauge needle
- Additional 96 well plate of adipocytes (cat# SA-1096)
- Tubes for dilution of standards

ASSAY PROCEDURE

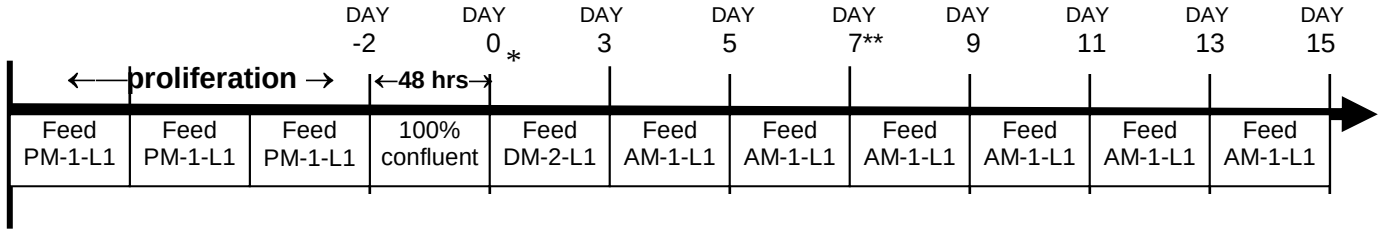
A. DIFFERENTIATION PROCEDURE

1. Preadipocytes are plated sub-confluent in 3T3-L1 Preadipocyte Medium (cat# PM-1-L1) and shipped the next day via overnight delivery.
2. Incubate cells until they are 100% confluent (in about 4-5 days). Cells will need to be fed every other day with PM-1-L1 during this time. See Table 1 for feeding volumes.
3. Once the cells are confluent, incubate an additional 48 hours before initiating differentiation.
4. Two days after the cells have been confluent, remove the Preadipocyte Medium (cat# PM-1-L1) and replace with an appropriate volume 3T3-L1 Differentiation Medium (cat# DM-2-L1; see table 1 below for recommended volumes). Incubate for 3 days.
5. Remove the 3T3-L1 Differentiation Medium and replace with 3T3-L1 Adipocyte Maintenance Medium. Incubate for 2-3 days.
6. Feed cells every 2-3 days using 3T3-L1 Adipocyte Maintenance Medium until ready for assay. 3T3-L1 adipocytes are suitable for most assays 7-14 days post differentiation (see Table 1 and 3T3-L1 Growth and Differentiation Feeding Schedule)

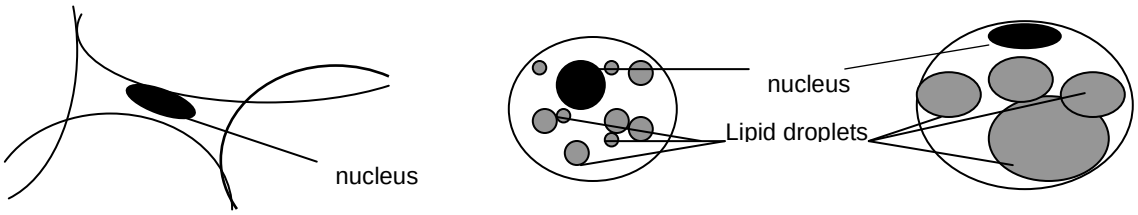
Table 1. Feeding Volumes

Format	Change PM-1-L1 to PM-1-L1		Change PM-1-L1 to DM-2-L1		Change DM-2-L1 to AM-1-L1		Change AM-1-L1 to AM-1-L1	
	OUT	IN	OUT	IN	OUT	IN	OUT	IN
96 well plate	90µl/well	90µl/well	150µl/well	150 µl / well	90 µl /well	120µl /well	90 µl /well	120µl /well
48 well plate	300 µl /well	300 µl /well	500µl /well	500 µl /well	300 µl /well	400 µl /well	300 µl /well	400 µl /well
24 well plate	0.6 ml/well	0.6 ml/well	1.0 ml/well	1.0 ml/well	0.6 ml/well	0.8 ml/well	0.6 ml/well	0.8 ml/well
12 well plate	1.2 ml/well	1.2 ml/well	2.0 ml/well	2.0 ml/well	1.2 ml/well	1.6 ml/well	1.2 ml/well	1.6 ml/well
6 well plate	1.8 ml/well	1.8 ml/well	3.0 ml/well	3.0 ml/well	1.8 ml/well	2.4 ml/well	1.8 ml/well	2.4 ml/well
T-75 flask	12 ml/flask	12 ml/flask	20 ml/flask	20 ml/flask	12 ml/flask	16 ml/flask	12 ml/flask	16 ml/flask
T-25 flask	4.2 ml/flask	4.2 ml/flask	7 ml/flask	7 ml/flask	4.2 ml/flask	5.6 ml/flask	4.2 ml/flask	5.6 ml/flask

3T3-L1 Growth and Differentiation Feeding Schedule



PREADIPOCYTE ⟶ *MATURE ADIPOCYTE*



* Once the cells are 100% confluent, incubate an additional 48 hours before initiating differentiation.

** 3T3-L1 adipocytes are suitable for most assays 7-14 days post differentiation

ASSAY PROCEDURE

1. Make your stock solution using whatever vehicle is appropriate for your test compounds. Dilute your stock solutions to their final concentration in LIP-2/3 Assay Buffer (100 ml is available). NOTE: if desired, maintain a constant concentration of solvent by preparing all compound dilutions in the highest concentration of that solvent. Dilute your controls in assay buffer. Prepare all vehicles as appropriate for your compounds, 0.1% DMSO has been included as the vehicle for the positive controls. Include the Assay Buffer alone as a vehicle control. PLEASE NOTE: ZEN-BIO DOES NOT RECOMMEND THE USE OF SOLVENTS AT CONCENTRATIONS ABOVE 1%.

2. Remove 120 µl medium from each well. Gently add 200 µl Wash Buffer to all wells. Remove 200 µl of the media and Wash Buffer from each well and replace with another 200 µl Wash Buffer.

3. Remove all the media and Wash Buffer from the cells from triplicate wells. Treat the cells with 150 µl of the test compounds resuspended in Assay Buffer three (3) wells at a time. Treat with the diluted Isoproterenol as positive control. Use the Assay Buffer alone as one of the vehicle controls. Please be sure to include both the vehicle provided in the kit and your vehicle (if your test compounds are not dissolved in DMSO). The assay should be performed in triplicate.

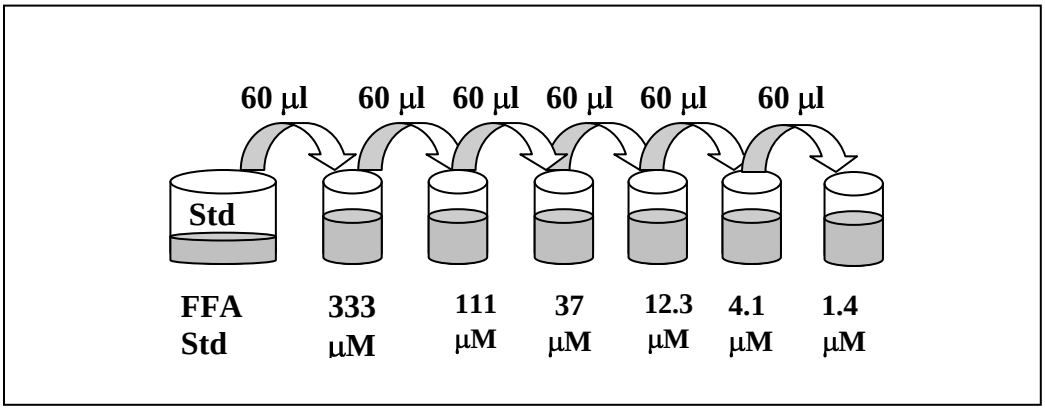
4. Incubate the plate at 37^{o}C-humidified incubator for 3 hours (for time course experiments the longest time point recommended is 5 hours). Note: Treatment times longer than 3 hours will result in significant fatty acid reutilization by the adipocytes and may decrease signal relative to total lipolysis activity.

A. DETECTION OF NON-ESTERIFIED FATTY ACIDS _____

1. Prepare the standard curve using the FFA STANDARD SOLUTION as follows:

   Briefly spin down the contents of the free fatty acid standard tube. Standards are: 0, 1.4, 4.1, 12.3, 37, 111, and 333 μM fatty acid. Prepare as follows:

   The kit standard solution is the 1.0 mM standard. Pipette 120 μl of Assay Buffer into 6 tubes (not provided). Pipette 60 μl of the FFA Standard Stock into a tube labeled 333 μM. Prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The Dilution Buffer alone serves as the zero standard.



Note: The above dilution series generates enough volume to perform the standard curve in duplicate. If you wish to perform the standard curve in duplicate, please note that seven fewer data points can be assayed with this kit.

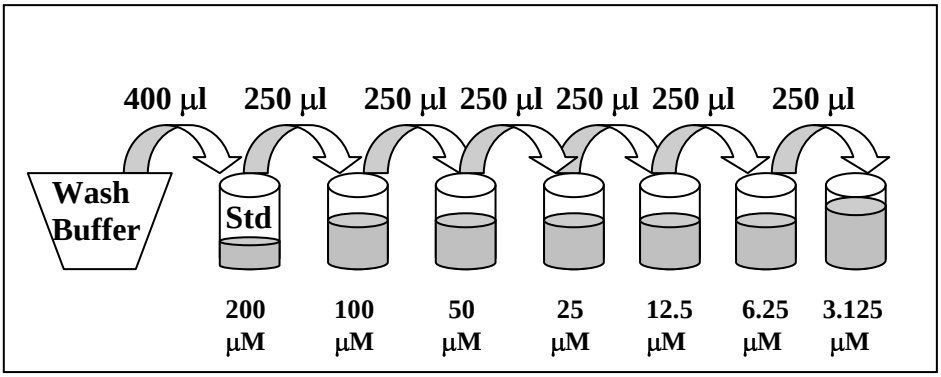
2. Add 10.5ml FFA Diluent A to the FFA Reagent A bottle and gently invert. DO NOT VORTEX! Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).
3. At the end of the incubation, 30 μl of the conditioned media is removed and transferred to the corresponding well of a blank plate for assessment of non-esterified fatty acids. [This is most easily accomplished using a multi-channel pipet.] Add 30 μl of each standard to empty wells.
4. Add the reconstituted FFA Reagent A to one of the disposable trays provided in the kit. Add 100 μl of FFA Reagent A to each well. Gently shake the plate to ensure mixing. Place in a 37 ^{o}C incubator for 10 minutes.
5. Add 5.5 ml FFA Diluent B to the FFA Reagent bottle and gently invert. Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).
6. Add the reconstituted FFA Reagent B to the other disposable tray provided in the kit. Add 50 μl of FFA Reagent B to each well. Gently shake the plate to ensure mixing. Place in a 37 ^{o}C incubator for 10 minutes.

7. Allow the plate to equilibrate to room temperature for 5 minutes. During this time, ensure that there are no bubbles in the solution mixture. Use a large gauge needle or clean pipet tip to pop any bubbles as this will result in inaccurate absorbance readings.
8. The optical density of each well is then measured at 540 nm.

B. DETECTION OF FREE GLYCEROL

1. One hour prior to the assay, prepare the glycerol standards as follows:

   Briefly spin down the contents of the glycerol standard tube before reconstitution. Pipette 400 µl of Wash Buffer into the 1 mM glycerol standard tube provided and mix well by vortexing. This produces a diluted stock glycerol standard of 200 µM. Pipette 250 µl of wash buffer into 6 tubes (not provided). Using the newly diluted stock glycerol solution, prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The 200 µM stock dilution serves as the highest standard, and the wash buffer serves as the zero standard.



Note: The above dilution series generates enough volume to perform the standard curve in duplicate. If you wish to perform the standard curve in duplicate, please note that eight fewer data points can be assayed with this kit.

2. Also at this time prepare the Glycerol Reagent A by adding 11.0 ml room temperature deionized water per bottle and gently invert. DO NOT VORTEX! Use a pipet to ensure that the powder is completely dissolved. Store at room temperature. If using a Reagent A solution previously prepared and stored at 2-8°C, also bring to room temperature. Make sure there is enough Reagent A from one solution to treat all the points in the assay. It may be necessary to combine solutions. Store in a light protected bottle. Reconstituted Glycerol Reagent A is stable for 7 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).
3. At the end of the incubation, an additional 100 µl of the conditioned media is removed and transferred to the corresponding well of a blank plate for assessment of free glycerol. [This is most easily accomplished using a multi-channel pipet. Add 100 µl of each glycerol standard to any remaining empty wells in one of the blank assay plates.

4. OPTION: to determine if the compound alone reacts with the Glycerol Reagent A, prepare a fresh plate (not included in kit) containing 50 µl of the compound. This plate can be incubated at 37°C with the treated cells. When performing the assay, add 50 µl of Glycerol Reagent A following the instructions in Steps 5 and 6.

5. Add the reconstituted Glycerol Reagent A solution to one of the disposable trays provided in the kit. Add 100 µl of Reagent A to each well of Plate B and Plate C (if used). Gently, pipet up and down once to mix. Pop the bubbles using a large gauge needle or a clean pipet tip. The plate is then incubated at 25°C (room temperature) for 15 minutes.

6. The optical density of each well is then measured at 540 nm.

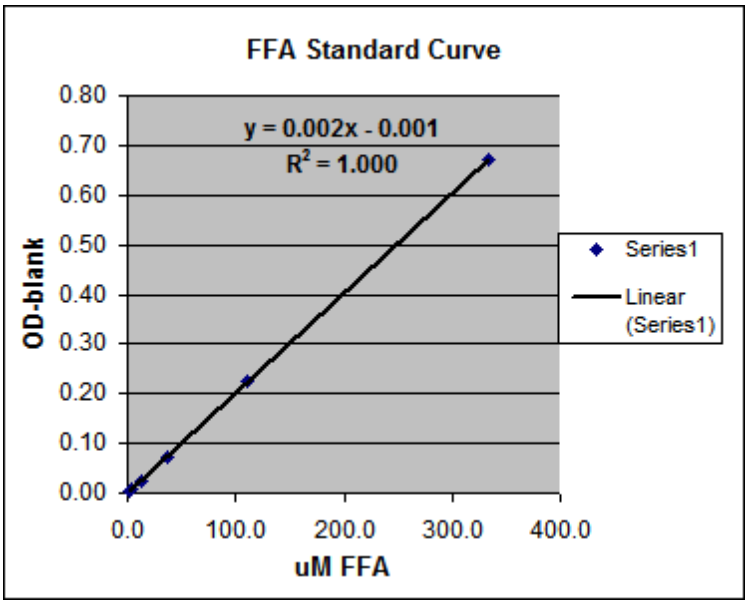
FATTY ACID STANDARD CURVE

Generate standard curve: see example below

[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the OD value of the 0μM standard from all OD values including the standard curve. . Note: 1mM standard is commonly omitted from analysis due to lack of linearity between 333 μM and 1mM. Optionally, a 4-parameter fit may be used to calculate standard curve.

uM FFA	OD	OD	OD-blank	OD-blank	Avg OD-blank
0	0.05	0.048			0.049
1.4	0.051	0.053	0.002	0.004	0.003
4.1	0.056	0.058	0.007	0.009	0.008
12.3	0.070	0.075	0.021	0.026	0.024
37	0.119	0.122	0.070	0.073	0.072
111	0.274	0.277	0.225	0.228	0.227
333	0.689	0.750	0.640	0.701	0.671



Slope	0.002
Intercept	-0.001
R^2	1.000

y = observed O.D. minus the blank
x = concentration of FFA in μM
To calculate x for each y, (i.e. to change the observed O.D. into FFA concentration) use the following equation:
y=(slope) times (x) plus intercept
y=mx+b so x=(y-b)/m
x=(y – (-0.001))/0.002 where 0.002= slope of the line and -0.001= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number.

Data are expressed as μM free fatty acids released.

OPTION: express data as Fold induction over appropriate vehicle

$$\text{Fold induction} = \frac{\mu\text{M free fatty acids SAMPLE}}{\mu\text{M free fatty acids VEHICLE}}$$

The R^2 value should be equal or greater then 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

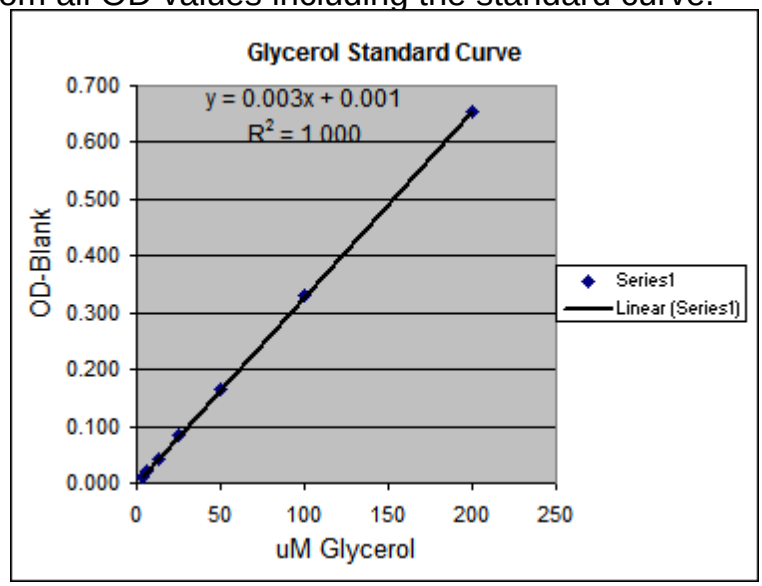
GLYCEROL STANDARD CURVE _____

Generate standard curve: see example below

[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the OD value of the 0μM standard from all OD values including the standard curve.

uM glycerol	OD	OD	OD-blank	OD-blank	Avg OD-blank
0	0.044	0.041			0.043
3.125	0.054	0.053	0.012	0.011	0.011
6.25	0.062	0.063	0.020	0.021	0.020
12.5	0.083	0.084	0.041	0.042	0.041
25	0.126	0.125	0.084	0.083	0.083
50	0.205	0.208	0.163	0.166	0.164
100	0.372	0.374	0.330	0.332	0.331
200	0.698	0.697	0.656	0.655	0.655



Slope	0.003
Intercept	0.001
R^2	1.000

y = observed O.D. minus the blank
x = concentration of glycerol in μM
To calculate x for each y, (i.e. to change the observed O.D. into glycerol concentration) use the following equation:

y=(slope) times (x) plus intercept

y=mx+b so x=(y-b)/m

x=(y – (0.001))/0.003 where 0.003= slope of the line and 0.001= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number.

Any OD values greater than the highest standard (200 μM) should be suspect. The compound should be re-assayed using a lower dose of the compound at treatment OR a dilute solution of the condition medium at the time of the assay.

The R^2 value should be equal or greater then 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

Data are expressed as μM glycerol released.

OPTION: express data as Fold induction over appropriate vehicle

$$\text{Fold induction} = \frac{\mu\text{M glycerol SAMPLE}}{\mu\text{M glycerol VEHICLE}}$$

APPENDIX A: PLATE LAYOUT _____

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

APPENDIX B: PROCEDURE FLOWCHART

ON DAY OF ASSAY

Make all test compound dilutions in Assay Buffer.

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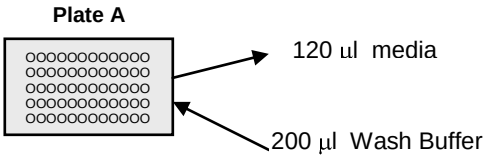
Remove 120 µl media from all wells. Add 200 µl Wash Buffer to all wells.



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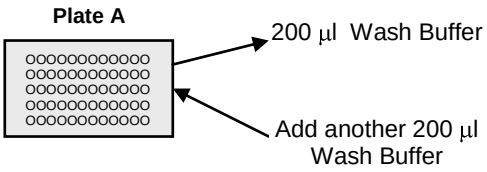
Remove 120 µl media & Wash Buffer. Add another 200 µl Wash Buffer to all wells.



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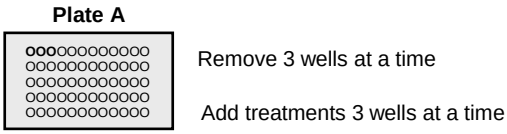
Remove all media & Wash Buffer. Add 150 µl treatments/controls to 3 wells at a time.



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Incubate 3-5 hours at 37°C.

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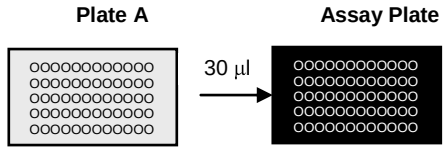
FREE FATTY ACID DETECTION

Remove 30 µl/well conditioned media from Plate A to Plate B. Add 30 µl FFA standards to empty wells



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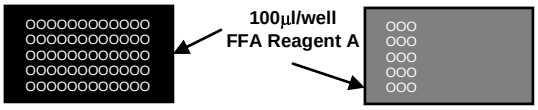
Reconstitute FFA Reagent A using Diluent A. Add 100µl/well. Incubate 10 minutes @ 37°C.



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Reconstitute FFA Reagent B using Diluent B. Add 50µl/well. Incubate 10 minutes @ 37°C.

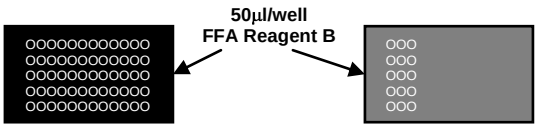


Plate C may be necessary for the assay of standards if al 96 wells of Plate A are used.

↓

Place at room temp. for 5 minutes. Pop any bubbles in each well using a clean pipet tip or large gauge needle.

↓

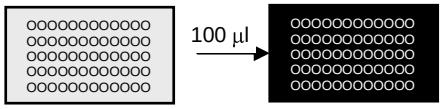
Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.

FREE GLYCEROL DETECTION

One hour prior to assay, reconstitute Glycerol Reagent A and prepare standards. Keep all at room temp.

↓

Remove 100 µl/well conditioned media from Plate A to a blank assay plate. Add 100 µl glycerol standards to empty wells.



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Add 100 µl/well reconstituted Glycerol Reagent A to a blank assay plate (including the glycerol standards at 50µl/well and optional plate without cells)

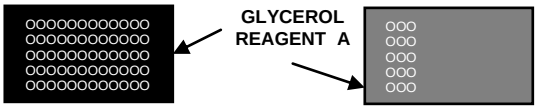

Plate C may be necessary for the assay of glycerol standards if al 96 wells of Plate A are used.

↓

Incubate at 25°C (room temperature) for 15 minutes. Pop the bubbles in each well.

↓

Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.

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